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(57) Abstract

The present invention describes a novel method of inducing immunity to cancer. This invention further discloses the use of Venezuelan Equine Encephalitis (VEE) virus vectors for expression of tumor–associated antigens, tumor–associated antigenic peptides and cytokines and methods for expressing these heterologous products in cultured cells, and in humans or animals.

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VENEZUELAN EQUINE ENCEPHALITIS VIRUS VECTORS EXPRESSING TUMOR-ASSOCIATED ANTIGENS TO INDUCE CANCER IMMUNITY

Priority

The present application claims priority under Title 35, United States Code, § 119 of United States Provisional Application Serial No. 60/068,080, filed December 18, 1997.

Field of the invention

The present invention describes a novel method of inducing immunity to cancer. This invention further discloses the use of Venezuelan Equine Encephalitis (VEE) virus vectors for expression of tumor-associated antigens, tumor-associated antigenic peptides and cytokines and methods for expressing these heterologous products in cultured cells, and in humans or animals.

Background of the invention

Cancer is a leading cause of death. Conventional treatment of cancer consists of chemotherapy, radiation therapy, and surgery, or a combination of these approaches. Novel and more effective strategies to combat cancer that eliminate the causes of these diseases, or to diminish pain and suffering, thereby prolonging the life of an affected patient are continually sought.

Tumor cells are known to express antigenic determinants that can be seen by the host's immune system. These determinants, called tumor-associated antigens (TAAs), are often altered or mutated forms of normal host cell proteins or peptides. They can be proteins or peptides that are not normally expressed in the tissue from which the tumor originates (Houghton, *J. Exp. Med.* 180: 1-4, 1994) and can act like a tag to identify tumor cells. One experimental approach to cancer therapy involves inducing the patient's immune system to mount an immune response against the tumor by attacking cells that express the TAAs.

The role of the immune system is to scan the body for proteins and other molecules that are not normally part of the body, called non-self or foreign antigens. If the immune system encounters a foreign molecule, an immune response is usually initiated. The immune response typically is divided into two branches. One branch is the humoral response in which B-cells are induced to produce antibodies against the foreign molecule. These antibodies can bind to the

molecules and promote a variety of biological activities. The second branch is the cellular branch in which cytotoxic T-cells are induced against the foreign molecule. The cytotoxic T-cells find other cells that express the foreign molecule and kill those cells. Once the immune system is stimulated, long-lived cells are expanded which specifically recognize the foreign antigen upon re-exposure to that antigen.

One method to induce the immune system to respond to a foreign peptide, protein or organism is to inject that foreign antigen into the body of the host by a process called vaccination. Typically, the peptide, proteins or organism is delivered to the host in a formulation that is conducive to stimulating the immune system. Should the host come in contact with the foreign antigen in the future, the foreign antigen will be more rapidly and vigorously eliminated by the immune system. This approach has been demonstrated to be useful in the infectious disease area where numerous vaccines have protected millions of individuals from viral and bacterial diseases. This experimental approach has met with little success in cancer therapy, however, when TAAs are injected. There are several reasons for the failure of this approach. These include the inability of the immune system to recognize the TAAs. Often, antibodies against the TAAs are induced in the patient, but these seem to be insufficient to alter the course of the disease. One explanation is that tumors may secrete factors responsible for dulling the immune response. What often appears to be lacking in the immune response against TAAs is a sufficient cellular response.

One reason for a lack of a sufficient cellular response is that the cytotoxic T-cells are not being stimulated appropriately. T-cells recognize TAAs and peptides derived from TAAs when these peptides are exposed on the surface of specialized cells that "present" the antigens to the T-cells. One class of antigen-presenting cell is called the dendritic cell (DC). DC progenitor cells originate in the hematopoetic system and reside in the skin and in the areas (interstitial spaces) surrounding cells in different tissues (Schuler and Steinman, *J. Exp. Med.* **186**: 1183-1187, 1997). DCs take up proteins and process them intracellularly to smaller peptides which are then transported to the cell surface in combination with a major histocompatibility complex (MHC) protein. It is the combination of appropriate accessory molecules on the cell surface together with the complex of the peptide in association with the MHC protein that serves to "present" antigen to the T-cells. The interaction of the DC and T-cell is thought to induce the production of cell stimulatory molecules, called cytokines, that stimulate the T-cells to respond to the presented TAA peptide.

An approach to stimulate the immune response to tumors has been to co-culture or "pulse" DCs with the TAA or related peptides *in vitro*. After the appropriate amount of time, the DCs are introduced back into the patient. Experimental evidence for this approach comes from Celluzzi et al., (*J. Exp. Med.* 183: 283-287, 1996), Paglia et al., (*J. Exp. Med.* 183: 317-322, 1996), and *Zitvogel et al.*, (*J. Exp. Med.* 183: 87-97, 1996) where experimental TAAs or peptides derived from them were co-cultured with DCs from a mouse. The DCs were reintroduced back into the mouse. The mouse was then challenged with tumor cells that expressed the TAA. Treatment with the *in vitro* pulsed DCs suppressed the growth of some tumors. Alternatively, the pulsed DCs were administered to a mouse that had an established tumor. In this case, transfer of the DCs lead to eradication of the tumors. Moreover, the animals were protected from subsequent challenge by the tumor cells.

A related approach to stimulate the immune response to tumors has been to express the TAA or peptides derived from TAAs in DCs *in vitro* by recombinant DNA techniques and then introduce these manipulated DCs into an animal that contains a tumor that expresses the TAA. For instance, Specht et al. (*J. Exp. Med.* 186: 1213-1221, 1997) infected DCs *in vitro* with a retrovirus that expresses the bacterial protein beta-galactosidase. The DCs expressed beta-galactosidase and presented beta-galactosidase-derived peptides on the cell surface. Mice were given tumor cells that express beta-galactosidase and dosed with the engineered DCs. There was a significant reduction in the number of pulmonary metastatic nodules compared to appropriate controls. In addition, Song et al., (*J. Exp. Med.* 186: 1247-1256, 1997) did a similar experiment in which beta-galactosidase was expressed in mouse DCs after infection of the DCs with an adenovirus vector that expresses beta-galactosidase. Introduction of the modified DCs into mice with beta-galactosidase-expressing tumors resulted in prolonged survival of the mice in both a prophylactic and therapeutic approach. Therefore, in these model systems, expression of the model TAA in DCs resulted in enhanced immunity against the tumor.

In addition to model systems, Hsu et al., (*Nature Medicine* 2: 52-58, 1996) isolated DCs from patients with B-cell lymphoma. The particular TAA was determined for each patient and was cocultured with the DCs *in vitro*. The DCs were then given back to the patients. All of the patients mounted an antitumor cellular response with three out of four patients showing clinical evidence of tumor regression. These data indicate that *in vitro* modification of DCs with the

appropriate TAA gives positive results in both experimental models and in clinical situations.

An improvement in the method of stimulating DCs to present the appropriate TAA would be beneficial in treating cancers. Current practices as outlined above involve isolating a patient's DCs and culturing the DCs *in vitro* with TAAs. This requires isolation of the TAAs and production in sufficient quantity to be administered to the cells. Other methods require infecting the DCs or their precursors with a retrovirus or adenovirus that expresses the TAA *in vitro*. Since retroviruses only integrate into dividing cells, only a smaller precursor population of DCs are targets for the therapy. This may limit the usefulness of this approach depending on the patient and the extent that these precursors are available. One alternative would be to use a system in which the TAA could be delivered directly to the DCs *in vivo*. Therefore, the DCs would not have to be isolated, expanded, treated *in vitro* and then re-introduced back into the patient.

Davis et al., (*J. Virol.* 70: 3781-3787, 1996) has used a live attenuated Venezuelan equine encephalitis (VEE) virus to protect mice from lethal challenge by influenza virus by expressing the influenza virus protein called hemagglutinin (HA). Interestingly, the HA antigen was expressed in the draining lymph node. It was postulated that high level expression in the lymph node by the VEE vector led to the strong induction of immunity.

In a subsequent seminar, R. Johnston showed evidence that DCs are infected *in vivo* by using a VEE that expresses the reporter protein, green fluorescent protein (GFP) (International Business Communication Meeting on Mammalian and Plant-based Expression Systems, September 22-23, 1997, Washington D.C.). Therefore, direct infection of mice with a VEE vector that expresses a targeted viral antigen results in expression in the lymph node in cells that appear to be DCs and results in strong immunity against a viral disease.

Using the same or similar VEE vector one could express a TAA in a cancer patient to stimulate an immune response against the TAA and the corresponding tumor. Since the VEE vector expressing the TAA would target the DC cells in the lymph node, one would anticipate an enhanced immune response against the tumor compared to a vector that did not target DCs. This could be done directly in an outpatient setting without the need to remove the DCs, culture them, expose them to TAA for various periods of time and then reintroduce them back into the patient.

Alternatively, one could still culture the DCs *in vitro* and use the natural affinity of the VEE vector for the DCs to deliver the TAAs. This would be a highly efficient *in vitro* method of expressing the TAAs and would be expected to stimulate a strong immune response when the modified DCs were administered back to the patient.

There are several additional approaches to generate the optimal immune response to the TAAs. In addition to the those mentioned above, the VEE expressing the TAA could be co-administered or sequentially administered either *in vivo* or *in vitro* with different cytokines to help stimulate the DCs and/or the T-cells to respond to the TAA. This co-administration could be in the form of another VEE expressing the cytokines or injection of purified cytokines. An example of a cytokine that stimulates the expansion of DCs is a synthetic molecule called progenipoietin-G (Streeter et al., *38th Annual American Society of Hematology Meeting*, San Diego, CA) In addition, optimal doses IL-2, IL-7 and IL-12 are known to enhance cytotoxic T lymphocyte responses (Kubo et al., US Patent 5,662,907). In addition, interferons alpha and beta and interleukin-15 may be valuable in stimulating the antitumor response. Synthetic cytokines based on interleukin-3, (WO 94/12638), chimeric fusion proteins between IL-3 and various growth factors (WO 95/21197 and WO 95/21254), and chimeric fusions between TPO and IL-3 (WO 97/12985) are also contemplated. Other peptides, such as those shown in PCT/US95/01000, PCT/US94/08672, WO 94/21287, US 5,674,486 can also be used. Alternatively, the VEE could express just peptides derived from TAAs in both the *in vivo* and *in vitro* situations. In addition, the TAA peptide may be made as a fusion with other peptides known in the art to stimulate the immune response including but not limited to peptides derived from tetanus toxin, *Plasmodium falciparum* CS protein, or the streptococcus 18 kilodalton protein. Moreover, more that one open reading frame may be expressed from the VEE vector by use of an appropriately placed internal ribosomal entry site between the first open reading frame and the second reading frame. In all situations, the patients may need more that one inoculation of VEE expressing the TAAs or of DCs that were modified by the VEE expressing the TAAs *in vitro* to see a clinical benefit. This boost to the immune response could come in the same form as the original treatment or by any other means. This includes another vector supplying the TAA or peptide, or injection of purified TAA or peptide in an appropriate adjuvant.

Summary of the invention

One object of the invention is a method of treating a subject who has cancer or who is at high risk of developing a tumor. The method comprises administering to the subject a recombinant Venezuelan equine encephalitis (VEE) virus that expresses an effective amount of heterologous substance from a heterologous nucleic acid segment, with the VEE virus containing at least one attenuating mutation. The heterologous nucleic acid segment comprises a promoter operable in the subject operatively associated with a nucleic acid encoding a tumor-associated antigen or peptide or cytokine effective in eliciting or enhancing an immune response to the tumor. The tumor-associated antigen(s) and related peptides can be taken from, but not limited to, the examples in the literature as reviewed by Houghton (*J. Exp. Med.* **180**: 1-4, 1994), Mumberg et al., (*Seminars in Immunology* **8**: 289-293, 1996) or Boon et al., (*Annu. Rev. Immunol.* **12**: 337-365, 1994) or from any other source including, but not limited to, public and private databases. Moreover, experimental methods of determining tumor-associated antigens are found in the literature, for example Hsu et al., (*Nature Med.* **2**: 52-58, 1996) or Kawakami et al., (*Proc. Nat. Acad. Sci. USA* **91**: 3515-3519, 1994; *Proc. Nat. Acad. Sci. USA* **91**: 6458-6462, 1994). Experimental methods of determining antigenic peptides of tumor-associated antigens are also found in the literature, for instance in Kawakami et al., (*Journal of Immunology* **154**: 3961-3968, 1995) and Celis et al., (*Proc. Nat. Acad. Sci. USA* **91**: 2105-2109, 1994). Examples of VEE vector sequences and manipulations are also found in the scientific and patent literature. These references include but are not limited to Johnston et al., (WO 95/ 32733), Davis et al., (US 5,185,440), Johnston et al., (WO 96/ 37616), Charles et al., (*Virol.* **228**: 153-160, 1997), Caley et al., (*J. Virol.* **71**: 3031-3038, 1997), Davis et al. (*J. Virol.* **70**: 3781-3787, 1996; *Vaccines* **95**: 387-391, 1995).

Another aspect of the invention is a DNA compromising a cDNA clone for an infectious Venezuelan equine encephalitis virus RNA transcript which contains at least one attenuating mutation and a heterologous promoter positioned upstream from the cDNA clone and operationally associated therewith. The VEE cDNA contains a heterologous promoter operably linked to a cDNA or coding region of a tumor associated antigen, the coding region of a peptide or peptides or the coding region or cDNA of a cytokine.

A further aspect of the invention is to isolate a patient's dendritic cells and culture them *in vitro* in an appropriate culture media supportive of growth and maintenance of the DC cell. The DC cells are infected *in vitro* with an infectious

recombinant Venezuelan equine encephalitis (VEE) virus with the VEE virus containing at least one attenuating mutation and a heterologous nucleic acid segment. The heterologous nucleic acid segment comprises a promoter operable in the subject operatively associated with a nucleic acid encoding a tumor-associated antigen or peptide or cytokine effective in eliciting or enhancing an immune response to the tumor. There are numerous references to the isolation and maintenance of DCs *in vitro*, for instance Hsu et al., (*Nature Medicine* 2: 52-58, 1996).

A further aspect of the invention is a method of treating a patient who has cancer or who is at risk of cancer with a viral vector that targets dendritic cells because the viral vector is a pseudotype virus which has the dendritic cell targeting properties of the Venezuelan equine encephalitis virus but may contain components of other viruses. The pseudotyped virus contains a heterologous nucleic acid segment which comprises a promoter operable in the subject operatively associated with a nucleic acid encoding a tumor-associated antigen or peptide or cytokine effective in eliciting or enhancing an immune response to the tumor.

A further aspect of the invention is a DNA that can be packaged into a pseudotyped viral particle that targets to the dendritic cell and contains a heterologous nucleic acid segment which comprises a promoter operable in the subject operatively associated with a nucleic acid encoding a tumor-associated antigen or peptide or cytokine effective in eliciting or enhancing an immune response to the tumor.

One aspect of the invention is a method of protecting or treating a subject against primary or metastatic neoplastic diseases, comprising: administering a recombinant Venezuelan Equine Encephalitis (VEE) virus vector to said subject in an effective amount, with said VEE virus vector containing at least one attenuating mutation, and with said VEE virus vector containing a heterologous DNA segment, said DNA segment comprising a promoter operably-linked to a DNA encoding a protein or peptide effective for treating said subject from said disease.

Preferably, the protein or peptide is a tumor-associated antigen. Even more preferably, the tumor-associated antigen is MAGE-1. Most preferably, the peptide has the amino acid sequence EADPTGHSY (SEQ ID NO: 4). Preferably, the protein or peptide is fused to another peptide selected from the group

consisting of QYIKANSKFIGITE (SEQ ID NO: 6), DIEKKIAKMEKASSVFNVVNS (SEQ ID NO: 7), and YGAVDSILGGVATYGAA (SEQ ID NO: 8).

Preferably, the heterologous promoter is Venezuelan equine encephalitis 26S subgenomic promoter.

Another aspect of the invention is a method wherein the administering step is a parental administration. Alternatively, the administering step is carried out by topically applying the virus to an airway surface of the subject. Alternatively, the administering step is an intranasal administration step. Alternatively, the administering step is an inhalation step.

Preferably, the protein is an adjunctive agent selected from the group consisting of natural cytokines and synthetic cytokines. Even more preferably, the cytokines are selected from the group consisting of interleukin-2, interleukin-7, interleukin-12, interleukin-15, interferon, and progenipoietin-G.

The invention is also directed to a method of modulating tumors in a patient with a VEE virus vector. It is also directed to a method of inhibiting proliferation of tumor cells with a VEE virus vector. Preferably, the tumor is selected from the group consisting of group consisting of lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

The invention is also directed to a process of inhibiting elevated numbers of tumor cells comprising administering to a host in need thereof a therapeutically effective amount of a VEE virus vector in unit dosage form. It is also directed to a process of treating primary or metastatic neoplastic diseases comprising administering to a mammalian host suffering therefrom a therapeutically effective amount of a VEE virus vector in unit dosage form.

The invention is also directed to a method of modulating primary or metastatic neoplastic diseases in a patient comprising the step of administering an effective amount of the protein or peptide to a patient. It is also directed to a method of inhibiting the production of tumor cells in a patient comprising the step of administering an effective amount of the protein or peptide to a patient.

Preferably, the tumor cell is characteristic of one selected from the group consisting of lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic

cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

The invention is also directed to a DNA comprising a cDNA clone coding for an infectious Venezuelan Equine Encephalitis (VEE) virus RNA transcript and a heterologous promoter positioned upstream from said cDNA clone and operatively associated therewith, and further comprising at least one attenuating mutation and containing the nucleotide sequence encoding a TAA, a TAA peptide or a natural or synthetic cytokine linked operably to a promoter.

The invention is also directed to a composition comprising a VEE virus vector wherein the protein or peptide is selected from the group consisting of tumor-associated antigens. Preferably, the tumor-associated antigen is MAGE-1. Even more preferably, the peptide has the amino acid sequence EADPTGHSY (SEQ ID NO: 4). Even more preferably, the protein or peptide is fused to another peptide having the sequence selected from the group consisting of QYIKANSKFIGITE, (SEQ ID NO: 6), DIEKKIAKMEKASSVFNVVNS, (SEQ ID NO: 7), or YGAVDSILGGVATYGAA, (SEQ ID NO: 8). Preferably, the composition includes an adjunctive agent selected from the group consisting of natural cytokines and synthetic cytokines. Even more preferably, the cytokine is selected from the group consisting of are interleukin-2, interleukin-7, interleukin-12, interleukin-15, interferon or progenipoietin-G.

The invention also is directed to an infectious VEE virus RNA transcript encoded by a cDNA clone of a recombinant VEE virus vector. It is also directed to infectious VEE virus particles containing such an RNA transcript.

The invention is also directed to a method of treating a subject against primary or metastatic neoplastic diseases by infecting a subject's dendritic cells with a composition comprising a recombinant VEE virus vector. It is also directed to a pharmaceutical formulation comprising infectious VEE particles in an effective amount in a pharmaceutically acceptable carrier.

The invention is also directed to an inoculum comprised of an effective amount of nucleic acid encoding a VEE virus vector dissolved or dispersed in an aqueous physiologically-tolerable or pharmaceutically-acceptable diluent. It is also directed to a pharmaceutical composition comprising a therapeutically effective amount of the VEE virus vector in admixture with a pharmaceutically acceptable carrier. It is also directed to a pharmaceutical composition comprising a VEE virus vector, further comprising an adjunctive agent, wherein said adjunctive agent is

selected from the group consisting of chemotherapeutic and immunotherapeutic agents.

The invention is also directed to a process for administering a VEE virus vector to protect or treat a subject against primary or metastatic neoplastic diseases, a process for inhibiting elevated numbers of tumor cells, a process for treating a host with an effective amount of VEE virus vector, and a process for treating a host an effective amount a tumor associated antigen, wherein the administering step in each of these processes is repeated.

Definitions

The term "VEE" means Venezuelan equine encephalitis.

The terms "TAA" and "TAAs" mean tumor-associated antigen and tumor-associated antigens, respectively.

The term "DC" and "DCs" mean dendritic cell and dendritic cells, respectively.

The term "DNA" means deoxyribonucleic acid.

The term "cDNA" means complementary deoxyribonucleic acid.

The term "RNA" means ribose nucleic acid.

The term "oligonucleotide" means a single-stranded sequence of nucleotides covalently bound in a 5' to 3' orientation.

The term "GFP" means green fluorescent protein.

The term "*in vitro*" means in a test tube or cell culture dish or container.

The term "*in vivo*" means in a body as opposed to in a test tube or culture dish.

The letters "g", "a", "t", and "c", in upper or lower case, when representing nucleotides, designate guanine, adenine, thymidine, and cytosine, respectively.

The term "peptide" means a series of amino acids connected to each other by amide bonds between the alpha-amino and carbonyl groups of adjacent amino acids. The length of the peptide is not critical and is typically less than about 30 amino acids.

The term "multimer" means one or more reiterations of a sequence of amino acids such as a peptide sequence covalently-linked together by amide bonds.

The term "vector" means any entity that transports a desired component to or in an appropriate host. For instance, a nucleic acid could be considered a vector when it is used to transport genetic information into a subject. Also, a virus particle may be considered a vector to transport proteins or nucleic acids in a host.

The term "PADRE" means any epitope which will bind multiple DR alleles. They are the alleles for Class II MHC ($CD4^+$ T helper cells recognize class II) - stimulate synthesis of IL-2 (growth factor) which then stimulates the CD^+ T cells (killer T cells or CTLs) among others. The PADRE, therefore, attaches to Class II MHC which then stimulates $CD4^+$ T cells which orchestrate a concerted immune response. The DR epitope is usually 12-25 amino acids.

Detailed description of the invention

The following examples will illustrate the invention in greater detail although it will be understood that the invention is not limited to these specific examples. Various other examples will be apparent to the person skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

General methods

General methods of cloning, expressing, and characterizing proteins are found in T. Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982, and references cited therein, incorporated herein by reference; and in J. Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, 1989, and references cited therein, incorporated herein by reference. DNA is typically purified from bacterial lysates using Promega Wizard Plus Minipreps or Qiagen Plasmid Midi or Maxi kits as per instructions. DNA fragments are isolated from agarose gel after electrophoresis using the Qiagen Qiaex II Gel Extraction Kit (Chatswsorth, CA).

All cell culture reagents can be obtained from Gibco/BRL Life Technologies (Gaithersburg, MD) unless otherwise noted. Baby hamster kidney (BHK-21) cells were used as a host cell for VEE vectors. There are obtained from ATCC (Rockville, MD) and are maintained in Dulbecco's modified Eagle's medium

supplemented with 5% fetal bovine serum (FBS). Dendritic cells are maintained in IMDM with 50 ug/ml gentamicin, 73 uM monothioglycerol (Sigma, St. Louis, MO), 20% FBS and various cytokines (see below). Restriction enzymes are available from Gibco/BRL Life Technologies (Rockville, MD), New England Biolabs (Beverly, MA), Promega (Madison, WI) or other suppliers. The restriction enzyme recognition sequences are also found in catalogues of suppliers of the enzymes. The standard genetic code can be found in various texts such as Metzler (Biochemistry: The Chemical Reactions of Living Cells, Academic Press, New York, 1977). Three letter and single letter codes for amino acids are also available (Metzler, supra).

Example 1. Modification of infectious Venezuelan equine encephalitis virus cDNA.

A molecular clone of VEE virus cDNA is available from ATCC as pV1003 (accession number 68013). This clone has the cDNA downstream from the bacteriophage T7 promoter for *in vitro* transcription of the viral genome. It was also shown by Davis et al., (US 5,185,440) that there is a 102 base deletion in the nsP3 gene but that RNA transcribed from this cDNA clone is infectious in cell culture and that the deletion mutant is also able to replicate in mice. In order to express a foreign gene, the VEE virus cDNA needs to have an internal promoter to direct expression of that foreign gene. Also useful is a unique restriction enzyme site into which the foreign gene can be easily inserted. Moreover, mutations need to be incorporated into the E2 glycoprotein gene to attenuate the virulence of the virus. Johnston et al., (WO 95/32733) demonstrate the insertion of a second copy of the 26S promoter sequences into an infectious VEE virus cDNA clone. Briefly, site-directed mutagenesis is used to convert wild-type sequences into the modified sequences. Several commercial kits are available for such purposes such as Promega's Altered Sites® Mutagenesis kit (Catalog #Q6210, Madison, WI). The nucleotide sequence of VEE virus is available in Genbank (accession L04653).

To add a second copy of the VEE virus 26S promoter into the genome, pV1003 is digested with *Eco*RI and *Hin*dIII. The approximately 4 kilobase fragment containing the 3' end of the genome can be subcloned into *Eco*RI and *Hin*dIII digested pAlter-1 (Promega) to yield plasmid pVEE1. Following the directions for mutagenesis that accompany the kit, an oligonucleotide containing the VEE virus 26S promoter is used to alter the VEE virus sequences. The oligonucleotide containing the 26S promoter sequences has the sequence:

aaacataattga/gaggggcccctataactctctacggctaacctgaatggactacgacatcgattaattaattaa/ata
ca gcagcaa (SEQ ID NO: 1)

where the nucleotides on the left are homologous to sequences just downstream (3') of the E1 gene in the 3' untranslated region of VEE virus. The sequences between the slashes contain the 26S promoter sequences, the unique *Cla*I site and an ochre translation termination codon in each forward reading frame. The sequences to the right of the second slash also are homologous to sequences just downstream (3') of the E1 gene in the 3' untranslated region of VEE virus. The resulting plasmid with the desired modifications is identified by *Cla*I digestion and nucleotide sequencing using fluorescent dye terminators using a Perkin Elmer ABI PRISM™ kit (Foster City, CA) and is designated pVEE2. To incorporate the changes back into the VEE virus genome, pVEE2 and pV1003 are digested with enzymes *Tth*111I and *Not*I. The approximately 3.9 kilobase pair sequence from pVEE2 is ligated into the larger fragment of pV1003 to yield a new plasmid pVEE3 which now has the second copy of the 26S promoter and a unique *Cla*I site.

Attenuation mutations in the E2 glycoprotein can be incorporated in the same manner as described above using pVEE1 as a target for oligonucleotide-directed mutagenesis. Attenuating mutations have been published by Johnston (WO 95/32733) and Davis (US 5,185,440) and can be accomplished by one skilled in the art using the Altered Sites® Mutagenesis kit as described. Examples of attenuating mutations are Glu76Lys, Thr120Lys, Glu209Lys or a combination of Glu209Lys and Lys245Asn (using the common three letter code for amino acids and the number indicating the amino acid number in the E2 glycoprotein). The amino acid to the left of the number is the amino acid in the wild type sequence and the amino acid to the right of the number is the amino acid in the attenuated strain at that location.

Example 2. Isolation of tumor associated antigens.

A number of TAAs can be introduced into a patient using the VEE virus vector system. One such antigen can be isolated from various cancer cells and is designated MAGE-1 (van der Bruggen et al., *Science* 254: 1643-1647, 1991). The MAGE-1 nucleotide sequence is available in Genbank, accession number M77481. To isolate the MAGE-1 cDNA, a cell line or tissue has to be identified that expresses the antigen. The human thyroid medullary carcinoma cell line TT is reported to express high levels of MAGE-1 (van der Bruggen et al., *Science* 254: 1643-1647, 1991). This cell line is available from ATCC (ATCC CRL 1803). Cells are cultured in Ham's F12 medium with 10% FBS and are plated at about 5×10^6

cells per T150 flask. Since the coding region for the MAGE-1 protein is not interrupted by introns, the nucleotide sequence can be isolated from cellular DNA. The are a number of commercially available kits for genomic DNA isolation from cells in culture. One is available from Qiagen. About 40 to 50 micrograms of genomic DNA are isolated from about 10^7 cells.

The polymerase chain reaction (PCR) (Saiki et al., *Science* **239**: 487-491, 1988) is utilized to isolate the MAGE-1 DNA sequences. The genomic DNA is diluted in sterile water to less than 10 micrograms per milliliter. A 1 microliter to 50 microliter aliquot of this diluted sample is used as a template in a PCR using a kit (GeneAmp) from Perkin-Elmer-Cetus (Norwalk, Conn) as per directions. For amplification of the sequence, two primers are synthesized by Genosys (Woodlands, TX) with the following sequence: the forward primer has the sequence:

atcgatgggtcttcattgcccagctcc (SEQ ID NO: 2)

where the first six nucleotides form the recognition sequence for *Cla*I and the remaining sequences are homologous to sequences untranslated and 5' to the coding region in exon 3. The reverse primer has the sequence

atcgattcccactggccttggctgcaac (SEQ ID NO: 3)

where the first six nucleotides form the recognition sequence for *Cla*I and the remaining sequences are homologous to the sequences untranslated at the 3' end of the coding region. PCR is carried out on a Perkin-Elmer-Cetus DNA Thermocycler at recommended conditions for 30 cycles. 10 microliters of the reaction are analyzed by agarose gel electrophoresis. After staining the gel with ethidium bromide, an approximately 1 kilobase band is present. The amplified DNA is cloned directly into a pGEM-T vector (Promega) as per instructions to yield plasmid pGEM-MAGE-1. DNA sequencing analysis verifies the correct sequence and distinguishes it from other related sequences such as MAGE-2 or MAGE-3 (van der Bruggen et al., *Science* **254**: 1643-1647, 1991). Because the amplified MAGE-1 DNA has *Cla*I sites at each end, it can be removed by *Cla*I digestion from pGEM-MAGE-1 and purified by agarose gel electrophoresis. The VEE virus vector, pVEE3, is digested with *Cla*I in the presence of shrimp alkaline phosphatase (US Biochemical, Cleveland, OH.). The alkaline phosphatase is inactivated by heating as per directions and the *Cla*I digested MAGE-1 sequence is ligated to pVEE3 using bacteriophage T4 DNA ligase. Correct orientation of the MAGE-1 DNA is determined by restriction enzyme mapping.

Example 3. Antigenic peptides and expression.

In addition to expression of the full length reading frame of MAGE-1 in the VEE virus vector, truncated regions of MAGE-1 may also be expressed. One example is to express a small peptide derived from MAGE-1 that is recognized by cytotoxic T lymphocytes. This small peptide has the amino acid sequence

EADPTGHSY (SEQ ID NO: 4)

using the single letter code for amino acids in the conventional amino terminus (on the left) to carboxyl terminus (on the right) designation (Kubo et al, US Patent 5,662,907). The oligonucleotide and its complement that encode this peptide are synthesized (Genosys, Woodlands, TX) with the following attributes: a *Cla*I site is synthesized at the immediate 5' end of the sequence followed by a methionine initiation codon, followed by the coding sequence of the following oligonucleotide:

gaagcagaccccaccggccactcctat (SEQ ID NO: 5)

followed by a translation termination codon and another *Cla*I restriction site. The strands are annealed together by heating equimolar amounts of the oligonucleotides in 100 microliter volumes together to boiling and letting the temperature return to room temperature. The annealed oligonucleotides are digested with *Cla*I and ligated to *Cla*I digested pVEE3 as described above. In addition to expressing just the monomeric peptide, the peptide sequences may be made into a multimers before ligating into the vector. For example, the peptide coding region (without the *Cla*I sites and the termination codon but with the initiator methionine codon at the 5' end) can be synthesized with an overlapping *Hin*dIII site at the 5' and the 3' ends. These are ligated together after annealing using T4 DNA ligase to form multimers whose size can be evaluated by polyacrylamide gel electrophoresis. Phosphorylated adapter molecules which include a *Cla*I site 5' of a overhanging *Hin*dIII site are ligated to the ends of the multimers. The multimers with the *Cla*I adaptors on the ends are digested with *Cla*I and ligated into pVEE3 as above.

Other versions of the MAGE-1 peptide can be synthesized by adding amino acid linker regions to the target peptide sequence. These linkers can be in the form of a string of alanine amino acids. The following oligonucleotide

(gaagcagaccccaccggccactcctat (SEQ ID NO: 5)

and its complementary sequence would be modified by incorporating the codons for alanine during the synthesis of the oligonucleotides in the desired number at either the 5' or 3' ends. A codon for the initiator methionine amino acid needs to be positioned appropriately at the 5' end of any designed oligonucleotide for proper translation. Multimers of SEQ ID NO: 5 with alanine linkers can be constructed using the same tactics described above. In addition to alanine, any other amino acid may be used to form a linker, preferably glycine or serine. Moreover, alternating or any other sequence arrangement of alanine and glycine and serine may be used.

The target peptide may also be expressed as a fusion with other peptides that stimulate the immune system. Examples of such peptides are sequences from tetanus toxin amino acid sequences 830-843

QYIKANSKFIGITE (SEQ ID NO: 6).

Plasmodium falciparum CS protein amino acid sequences 378-398

DIEKKIAKMEKASSVFNVVNS (SEQ ID NO: 7),

or streptococcus 18 kilodalton protein amino acids 1-16

YGAVDSILGGVATYGAA (SEQ ID NO: 8).

These peptides may be fused at the amino terminal or carboxyl terminal end of the MAGE-1 peptide epitope by conventional molecular biology techniques as described above. In addition, various sized linkers such as alanine, serine and/or glycine amino acids can be used to space the peptide sequences to a desired distance.

Example 4. Isolation and cloning of a cytokine sequence into a VEE virus vector.

Certain cytokines are known to stimulate the immune response. Therefore, it may be beneficial to express a cytokine from the VEE vector. One such cytokine is interleukin-2 (IL-2) (Taniguchi et al., *Nature* 302: 305-310, 1983). A plasmid that contains the human IL-2 cDNA is available from ATCC (pLW81, ATCC number 61390). The nucleotide sequence of the human IL-2 coding region is available in Genbank (accession number A06759). Plasmid pLW81 can be used as a template using PCR to generate the IL-2 coding region with *Cla*I ends. The forward primer has the sequence:

atcgat/aacctcaactcctgccaca (SEQ ID NO. 9)

in which nucleotides to the left of the slash form a *Cla*I restriction site and the nucleotides to the right of the slash are homologous to the 5' untranslated region of the gene. The reverse primer has the sequence:

atcgat/agtgggaagcacttaattat (SEQ ID NO. 10)

in which nucleotides to the left of the slash form a *Cla*I restriction site and the nucleotides to the right of the slash are homologous to the 3' untranslated region of the gene. The PCR product is ligated into pGEM-T as described above to form pGEM-IL-2. After confirmation of the correct nucleotide sequence by DNA sequencing, the IL-2 gene is removed from pGEM-IL-2 by *Cla*I digestion and ligated into pVEE3 as described above.

Example 5. Isolation and culturing of human dendritic cells.

Human DCs are accessible from either cord blood (Romani et al., *J. Exp. Med.* 180: 83-93, 1994) or from bone marrow (Szabolcs et al., *J. Immunology* 154: 5851-5861, 1995). Briefly, bone marrow cells are centrifuged in standard Ficoll-Hypaque gradients and the mononuclear cells are isolated from the interface. These cells are washed twice with phosphate buffered saline/0.1% bovine serum albumin, adjusted to 10^6 cells per milliliter and kept on ice for 30 minutes in the presence of 50 microgram per milliliter mouse anti-CD34 monoclonal antibody (Oncogene Science, Uniondale, NY). The cells are washed three times to remove unbound monoclonal antibody and incubated with sheep anti-mouse IgG1 immunomagnetic beads (Dynal, Oslo, Norway) for 30 minutes on ice. CD34 positive cells were collected by using a magnet. The beads are detached from the cells by incubation overnight in IMDM/20% FBS without enzyme. Suspension cultures of the CD34+ cells are started in IMDM/20% FBS at 2 x 10^5 cells per 5 milliliters in a 35 mm tissue culture dish supplemented with 20 nanograms per milliliter c-kit ligand (Amgen, Thousand Oaks, CA), 100 nanograms per milliliter GM-CSF (Sandoz, East Hanover, NJ) and 10 to 100 nanograms per milliliter TNF-alpha (Genentech, San Francisco, CA). The DCs ($CD14^-$ and $HLA\text{-}DR^+$) are identified using a panel of monoclonal antibodies to CD14/LeuM3 and HLA-DR (from Becton Dickinson, San Jose, CA).

Example 6. Generation of infectious virus from VEE virus vectors.

Plasmid pVEE3 and derivatives are digested with *Not*I to linearize the plasmid and run-off transcripts are generated by T7 RNA polymerase as described (Davis et al., US Patent 5,185,440). Transcripts are transfected into BHK-21 cells

as described (Polo et al., *Journal of Virology* 62: 2124-2133, 1988) using DEAE-Dextran (Sigma, St. Louis, MO) although electroporation and transfection by liposome reagents are also acceptable. After about 24 to 60 hours, when cytopathic effects have peaked, the supernatant is harvested. The quantity of infection virus is determined by standard plaque assays or infectivity assays in BHK-21 cells (Burleson et al., *Virology: A Laboratory Manual*, Academic Press, San Diego, 1992). Virus is expanded on BHK-21 cells and further purified by sucrose gradient techniques using endotoxin free sucrose. Briefly, cells are infected using a multiplicity of infection (MOI) of 1 infectious unit of virus per cell. The supernatant is aspirated from the cells and overlayed with a minimal volume of virus such that the cells are just submerged. After 30 to 60 minutes at 37°C with occasional rocking to prevent drying, an appropriate volume of medium is added to the cells for normal culturing. Virus is harvested when cytopathic effects have peaked.

Alternatively, dendritic cells can be infected at a lower MOI between 0.001 and 01. After absorption of the virus, the cells are washed three times in phosphate-buffered saline with 5% autologous serum and administered back to the host by intravenous infusion over an hour period.

Example 7. Inoculation of VEE virus vectors into a host.

Six week old female Balb/c mice (Charles Rivers Laboratories, Wilmington, MA) are put under light Metofane anesthesia (Pitman-Moore). They are inoculated in the rear footpad with an appropriate amount of virus inoculum, preferably about 10^4 infectious units, suspended in a 10 microliter volume of phosphate-buffered saline containing 5% donor calf serum(Caley et al, *Journal of Virology*, 71: 3031-3038, 1997). Reinoculation or "boosting" with a given virus is given at timed intervals such as 3 weeks using approximately 10^5 infectious units per footpad. For nasal inoculation, the mice are anesthetized and a pipetman is use to deliver 5-10 ul of inoculum to the nares. For intraperitoneal inoculations, the virus is diluted as above and 10^2 to 10^7 infectious units are delivered with a 27-guage needle. Serum samples can be obtained from tail vein bleeds at various times post-inoculation or post-boosting to determine antibody titers.

All references, patents, or applications cited herein are incorporated by reference in their entirety as if written herein.

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SEQ ID Correlation Table

SEQ ID NO:	Sequence	Description/Name
(SEQ ID NO: 1)	aaacataattga/gaggggcccctataac tctctacggctaacctgaatggactacga catcgattaattaattaa/atacagcagc aa	
(SEQ ID NO: 2)	atcgatgggtcttcattgcccagctcc	
(SEQ ID NO: 3)	atcgattcccactggccttggctgcaac	
(SEQ ID NO: 4)	EADPTGHSY	
(SEQ ID NO: 5)	gaagcagaccccaccggccactcctat	
(SEQ ID NO: 6)	QYIKANSKFIGITE	
(SEQ ID NO: 7)	DIEKKIAKMEKASSVFNVVNS	*Plasmodium falciparum* CS protein amino acid sequences 378-398
(SEQ ID NO: 8)	YGAVDSILGGVATYGAA	streptococcus 18 kilodalton protein amino acids 1-16
(SEQ ID NO. 9)	atcgat/aacctcaactcctgccaca	
(SEQ ID NO. 10)	atcgat/agtgggaagcacttaattat	

Claims

1. A method of protecting or treating a subject against primary or metastatic neoplastic diseases, comprising:

administering a recombinant Venezuelan Equine Encephalitis (VEE) virus vector to said subject in an effective amount, with said VEE virus vector containing at least one attenuating mutation, and with said VEE virus vector containing a heterologous DNA segment, said DNA segment comprising a promoter operably-linked to a DNA encoding a protein or peptide effective for treating said subject from said disease.

2. A method according to claim 1, wherein said protein or peptide is a tumor-associated antigen.

3. A method of claim 2 in which said tumor-associated antigen is MAGE-1.

4. A method of claim 1 in which said peptide has the amino acid sequence EADPTGHSY (SEQ ID NO: 4).

5. A method of claim 1 in which the said protein or peptide is fused to another peptide selected from the group consisting of

QYIKANSKFIGITE (SEQ ID NO: 6),

DIEKKIAKMEKASSVFNVVNS (SEQ ID NO: 7), and

YGAVDSILGGVATYGAA (SEQ ID NO: 8).

6. A method according to claim 1, wherein said heterologous promoter is Venezuelan equine encephalitis 26S subgenomic promoter.

7. A method according to claim 1, wherein said administering step is a parental administration.

8. A method according to claim 1, wherein said administering step is carried out by topically applying said virus to an airway surface of said subject.

9. A method according to claim 1, wherein said administering step is an intranasal administration step.

10. A method according to claim 1, wherein said administering step is an

inhalation step.

11. A method of claim 1, wherein said protein is an adjunctive agent selected from the group consisting of natural cytokines and synthetic cytokines.

12. A method of claim 11, wherein said cytokines are selected from the group consisting of interleukin-2, interleukin-7, interleukin-12, interleukin-15, interferon and progenipoietin-G.

13. A method of modulating tumors in a patient with a VEE virus vector of claim 1.

14. A method of inhibiting proliferation of tumor cells with a VEE virus vector of claim 1.

15. A method of claim 13 and 14 wherein said tumor is selected from the group consisting of group consisting of lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

16. A process of inhibiting elevated numbers of tumor cells comprising administering to a host in need thereof a therapeutically effective amount of a VEE virus vector of claim 1 in unit dosage form.

17. A process of treating primary or metastatic neoplastic diseases comprising administering to a mammalian host suffering therefrom a therapeutically effective amount of a VEE virus vector of claim 1 in unit dosage form.

18. A method of modulating primary or metastatic neoplastic diseases in a patient comprising the step of administering an effective amount of the protein or peptide as recited in claim 2 to said patient.

19. A method of inhibiting the production of tumor cells in a patient comprising the step of administering an effective amount of the protein or peptide as recited in claim 2 to said patient.

20. The method of claims 16, 17, 18 or 19 wherein the tumor cell is characteristic of one selected from the group consisting of lung cancer, breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, gastric cancer, colon cancer, renal cancer, bladder cancer, melanoma, hepatoma, sarcoma, and lymphoma.

21. A DNA comprising a cDNA clone coding for an infectious Venezuelan Equine Encephalitis (VEE) virus RNA transcript and a heterologous promoter positioned upstream from said cDNA clone and operatively associated therewith, and further comprising at least one attenuating mutation and containing the nucleotide sequence encoding a TAA, a TAA peptide or a natural or synthetic cytokine linked operably to a promoter.

22. A composition according to claim 21, wherein said protein or peptide is selected from the group consisting of tumor-associated antigens.

23. A composition of claim 21 in which said tumor-associated antigen is MAGE-1.

24. A composition of claim 21 in which said peptide has the amino acid sequence EADPTGHSY (SEQ ID NO: 4).

25. A composition of claim 21 in which said protein or peptide is fused to another peptide having the sequence

QYIKANSKFIGITE, (SEQ ID NO: 6),

DIEKKIAKMEKASSVFNVVNS, (SEQ ID NO: 7), or

YGAVDSILGGVATYGAA, (SEQ ID NO: 8).

26. A composition of claim 21, wherein said protein is an adjunctive agent selected from the group consisting of natural cytokines and synthetic cytokines.

27. A composition of claim 26, wherein said cytokines are interleukin-2, interleukin-7, interleukin-12, interleukin-15, interferon or progenipoietin-G.

28. An infectious VEE virus RNA transcript encoded by a cDNA clone of claim 21.

29. Infectious VEE virus particles containing an RNA transcript of claim 28.

30 A method of treating a subject against primary or metastatic neoplastic diseases by infecting a subject's dendritic cells with a composition of claim 29.

31. A pharmaceutical formulation comprising infectious VEE particles

according to claim 29 in an effective amount in a pharmaceutically acceptable carrier.

32. An inoculum comprised of an effective amount of nucleic acid encoding claim 21 dissolved or dispersed in an aqueous physiologically-tolerable or pharmaceutically-acceptable diluent.

33. A pharmaceutical composition comprising a therapeutically effective amount of the compound according to claim 1 in admixture with a pharmaceutically acceptable carrier.

34. A pharmaceutical composition of claim 1 further comprising an adjunctive agent, wherein said adjunctive agent is selected from the group consisting of chemotherapeutic and immunotherapeutic agents.

35. The process according to claims 1, 16, 17, 18, or 19 wherein said administering step is repeated.

Sequence Listing

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

Intern. .tal Application No
PCT/US 98/25725

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 24447 A (VIAGENE INC) 10 July 1997 see page 2, line 5 - page 3, line 4 see page 8, line 29 - page 9, line 5 see page 17, line 3-8 see page 14, line 1-7 see page 20, line 11 - page 21, line 18 see example 2 see claims	1-3,7, 11-17, 20-23, 26,27, 32-35
Y	--- -/—	4-6, 8-10,18, 19,24, 25,28-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 May 1999	18/05/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter. ...nal Application No
PCT/US 98/25725

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 04542 A (SIDNEY JOHN C ;CYTEL CORP (US); FIKES JOHN D (US); LIVINGSTON BRIA) 16 February 1995 see page 3, line 4-15 see page 21, line 19-21 see page 12, line 24 - page 14, line 3 see seq.ID 20	5,18,19, 25
Y	WO 95 32733 A (UNIV NORTH CAROLINA) 7 December 1995 see example 1 see page 2, line 17 - page 3, line 2	6,8-10, 28-31
Y	WO 95 20974 A (SLOAN KETTERING INST CANCER ;LUDWIG INST CANCER RES (US)) 10 August 1995 see page 20 seq.ID 4 see claim 12	4,24
X	BROSSART P ET AL.: "Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL" J.IMMUNOL., vol. 158, 1997, pages 3270-3276, XP002102050 see abstract see page 3270, left-hand column, line 1 - right-hand column, line 14 see page 3271, right-hand column, line 6-28 see page 3275, right-hand column, line 18 - page 3276, left-hand column, line 26	1,2,7, 13-20, 33-35
A	RUSSO, V. ET AL: "Transfer of tumor-associated antigen genes into dendritic cells for activ immunotherapy of neoplastic diseases." BLOOD, (NOV. 15, 1997) VOL. 90, NO. 10 SUPPL. 1 PART 1, PP. 405A. MEETING INFO.: 39TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY SAN DIEGO, CALIFORNIA, USA DECEMBER 5-9, 1997 THE AMERICAN SOCIETY OF HEMATOLOGY. ISSN: 0006-4971., XP002101280 see the whole document	1-35
A	TUTING T ET AL: "Gene-based strategies for the immunotherapy of cancer." JOURNAL OF MOLECULAR MEDICINE, (1997 JUL) 75 (7) 478-91. REF: 142 JOURNAL CODE: B8C. ISSN: 0946-2716., XP002101281 GERMANY: Germany, Federal Republic of see page 484, left-hand column, paragraph 3 - page 487, right-hand column	1-35

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/US 98/25725

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,A	TUTING T ET AL: "Autologous human monocyte-derived dendritic cells genetically modified to express melanoma antigens elicit primary cytotoxic T cell responses in vitro: enhancement by cotransfection of genes encoding the Th1-biasing cytokines IL-12 and IFN-alpha." JOURNAL OF IMMUNOLOGY, (1998 FEB 1) 160 (3) 1139-47. JOURNAL CODE: IFB. ISSN: 0022-1767., XP002102051 United States	1-35
P,A	WO 98 08947 A (US HEALTH) 5 March 1998 see page 8, line 34 - page 10, line 33	1,11,12, 21,26,27

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/25725

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-20, 30, 35
because they relate to subject matter not required to be searched by this Authority, namely:
See FURTHER INFORMATION SHEET PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Although claims 1-20 30 35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Claims Nos.: 1-20 30 35

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Intern ...al Application No
PCT/US 98/25725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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